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Cinisello Balsamo, 15th March 2005

This document contains the results from a series of *in vitro* and *in vivo* experiments which were performed to compare the pharmacological properties of example D1/12 which is the lead compound from the series of derivatives described in WO97/43251 (D1) and examples 01, 03, 04, 05 and 15 from the present patent application.
In the case of examples 01, 03, 05 and 15 the comparison with D1/12 is restricted to one *in vitro* test only whereas 04 and D1/12 are compared in a larger number of assays. This is due to our internal screening procedure which is based on initial cytotoxicity and *in vitro* efficacy (inhibition of LPS-induced TNFα production in human peripheral blood cells) assays followed by additional *in vitro* and *in vivo* tests for those compounds which have similar/identical efficacy to D1/12 but reduced cytotoxic effect.

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EXHIBIT 1

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IN VITRO CYTOTOXICITY TEST

The cytotoxicity of the test compounds was evaluated by a colorimetric assay using a commercial kit. The kit determines the number of viable cells according to their ability to metaboilize a tetrazolium salt which produces formazan. The amount of formazan produced is proportional to the number of viable cells. The Hep-G2 human hepatoma cell line was used for the cytotoxicity test.

HepG2 cells are incubated in the presence or the absence of the test compounds at the final concentration of 10^{-6} M. The test is performed in triplicate. After 48h incubation the supernatant is removed and the optical density at 490 nm is determined after 30, 60 and 90 min at 37°C using a multiscan plate reader. The 60 min reading is generally used to calculate the growth rate.

The results (Table 1) show that at the concentration of 10-6 M the example D1/12 is far more cytotoxic than the other test examples.

Table 1. Hep-G2 CYTOTOXICITY TEST

	Growth Inhibition *versus* Control (%)
Concentration	10^{-6} M
Example D1/12	31.5
Example 01	3.8
Example 03	7.3
Example 04	0
Example 05	0
Example 15	0

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HUMAN MK-CFU COLONY FORMATION

In this test, human precursors of bone marrow megacarocytes are grown in vitro with the appropriate medium and growth factors and their maturation is measured as the ability of matured cells to form colonies. The ability of a given compound to inhibit the formation of colonies is an index of its toxic potential on the bone marrow.
Human cell precursors were obtained from Human Cord Blood (Cambrex). Cell suspension was divided in tubes containing cytokines, collagen and testing compounds in a range of concentration between 10 and 1000 nM. Cell suspension were plated in duplicate on chamber slides and incubated for 12 days in humid chambers at 37° and 5% CO_2. At the end of the incubation period the slides were dehydrated, fixed and stained with an antibody specific for $GP_{IIb/IIIa}$. The number of megakaryocyte colony was determined by light microscope counting.

The results of this test using compounds D1/12 and 04 are shown in Table 1.
As in the previous test of cytotoxicity on HepG2 cells, D1/12 was more toxic than 04 also in the test of colony formation.

Table 1. Inhibition of HUMAN Mk-CFU colony formation

	Mk-CFU development IC_{50} (nM)
D1/12	48
04	185

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In vitro metabolic stability in rat and human S9 fraction

The *in vitro* metabolic stability of the test compounds was investigated in rat and human S9 fractions, a pool of hepatic enzymes which are known to transform drugs *in vivo*. The compounds (5 µg/mL each) were incubated with rat and human S9 fractions (protein content 2 mg/mL) in potassium phosphate buffer (pH 7,4) at 37°C for 0, 10, 30, 60 and 90 min. The reaction was stopped by placing the test tubes in an ice bath and by adding acetonitrile with 0.2%TFA. The mixture was mixed vigorously then centrifuged at 12,000 rpm for 5 min. Aliquots of the supernatant were injected into a HPLC-UV system. The percentage of un-transformed compound at the various times of incubation was thus measured. The results are shown in Tables 1 and 2.
As can be seen 04 is more metabolically stable than D1/12 in S9 from both species.

Table 1. Amount of 04 and D1/12 (expressed as % of the initial concentration) found after incubation of the test compounds at 37°C in rat S9 fraction.

Compound	Time (min)			
	10	30	60	90
O4	95.2	89.2	84.7	84.0
D1/12	37.6	10.6	7.1	5.8

Table 2. Amount of 04 and D1/12 (expressed as % of the initial concentration) found after incubation of the test compounds at 37°C in human S9 fraction.

HUMAN S9 FRACTION				
Compound	Time (min)			
	10	30	60	90
O4	99.7	96.1	94.4	94.0
D1/12	96.5	90.0	85.7	81.5

LPS-induced TNFα production in the mouse

The efficacy of examples 04 and D1/12 as inhibitor of TNFα production was evaluated in the mouse. The model used is considered predictive for human septic shock.
Briefly, mice (CD-1, female, 20-22 g, 10 animals/group) were treated intraperitoneally with a lethal dose of LPS and 90 minutes later TNFα level in the serum was determined by a commercially available ELISA kit.
Examples 04 and D1/12 (1 mg/Kg) were administered orally by gavage as a methyl cellulose suspension 60 minutes before LPS. The results are reported in the following table.

Table 1. Effect of examples 04 and D1/12 on LPS-induced serum TNFα in the mouse

Treatment	TNFα (ng/ml)	Inhibition (% vs Control)
LPS (Control)	8.14±1.99	-
Example 04 1 mg/Kg	2.38±0.61	71
Example D1/12 1 mg/Kg	3.13±1.56	62

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TNBS-induced colitis in the rat

In this model, rectal instillation of trinitrobenzene sulfonic acid (TNBS) leads to an immunological response against mucosal cells characterized by a severe colonic inflammation. The animals develop colitis with a reduced intestinal function and absorption. At the end of the experiment (day 7 after colitis induction) the intestine of the affected animals appears severely damaged with macroscopic ulcers. In this model several parameters are monitored throughout the experiment to describe the development and the severity of the colitis. They are:

a) Rate of growth of the animals: the body weight gain during the experiment is monitored and compared to that of a group of naive animals treated with vehicle.
b) Colon damage: represents the gravity of the damage and is quantified by a score determined at the necropsy.
c) Colon weight: represents the overall inflammation state of the intestine since during colitis the tissue becomes more oedematous and is infiltrated by inflammatory cells
d) Stool consistency: liquid stools (diarrhoea) is a consequence of development of colitis.
e) Mucosal TNFα: during development of colitis the infiltrated inflammatory cells produce this cytokine that sustains and amplifies inflammation. The level of TNFα is determined by commercial ELISA in the tissue extract from the intestine.
f) Mucosal myeloperoxidase (MPO): MPO is a specific marker of polymorphonuclear cells that represent the major population of inflammatory cells in an affected tissue. The level of tissue MPO content is a direct measure of the cell infiltration and is carried out using an enzymatic assay.

Animals (Sprague Dawley rats, male, 150-175 g, 6 animals/group) were treated orally with example 04 or example D1/12 at the dose of 1 mg/Kg. Both drugs were administered daily by gavage as a Methyl Cellulose suspension immediately after TNBS instillation. Daily treatment continued for the entire duration of the experiment (7 days)
The results are reported in Table 2 and expressed as percentage of the control (colitis) animals.

Table 2. Effect of 04 and D1/12 on TNBS-induced colitis in the rat

Treatment (1 mg/kg)	Body weight gain (% gain)	Colon damage (% inihib.)	Colon weight (% inhib)	Stool consistency (% improv.)	Mucosal TNFα (% inhib)	Mucosal MPO (% inhib)
Example 04	34	88	65	100	74	75
Example D1/12	11	17	14	20	20	32

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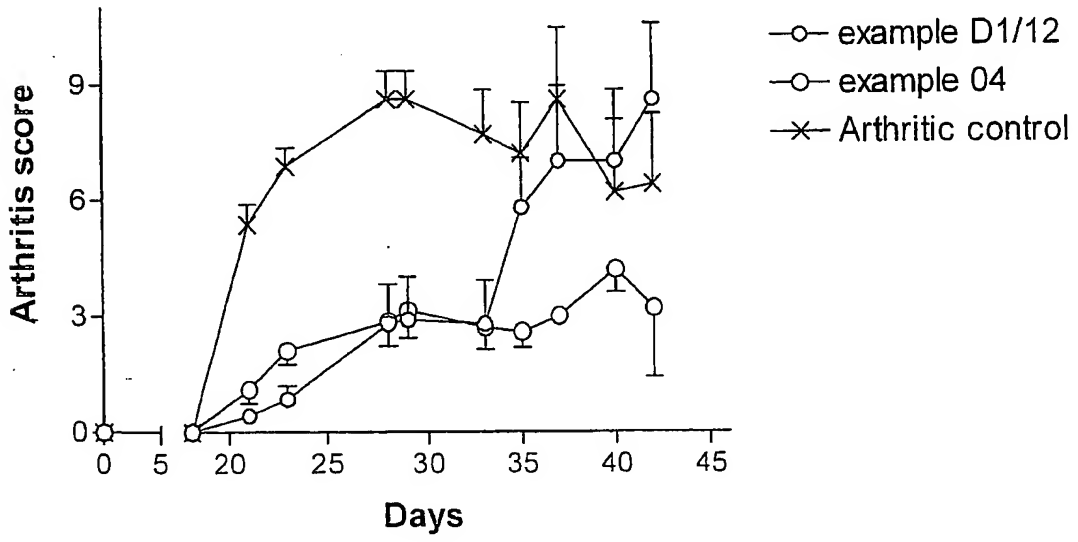
Collagen-induced arthritis (CIA) in the mouse

In this model a poly-articular arthritis is induced by s.c. administration of bovine Type II collagen in Complete Freund's Adjuvant followed by a second administration of collagen in incomplete Freund's Adjuvant after 21 days. After the second administration of collagen, animals develop arthritis of increasing severity in both hind and rear paws. The CIA model is extensively used to study new anti-arthritic drugs since it is believed to resemble human rheumatoid arthritis.
The development of arthritis is determined by examining each paw during the entire experiment and the severity of the disease is quantified by a score based on the presence of hyperhoemia, oedema and the number of affected joints. The score ranges from 0 to 4 for each paw, leading to a maximal value of 16 for each animal and is determined as follows:

Score 0, naïve conditions
Score 1, mild but definitive redness or swelling of the ankle or wrist
Score 2, moderate redness and swelling of the ankle or wrist
Score 3, severe redness of the entire paw including digits
Score 4, maximally inflamed limb with involvement of multiple joints

In the present study, animals (DBA/1 mice, male, 20-22 g, 5-15 animals/group) were treated orally with example 04 or example D1/12 at the dose of 10 mg/Kg. Both drugs were administered daily by gavage as a methyl cellulose suspension.
The results are summarized in Figure 1 where the progression of the clinical score is shown.



As can be seen, whereas D1/12 and 04 are similarly effective during the first 33 days of treatment, only 04 continues to exert the same level of efficacy after day 33. In contrast after day 33, animals receiving D1/12 have clinical score statistically indistinguishable from the arthritic controls.